Pretreatment with a 55-kDa Tumor Necrosis Factor Receptor–Immunoglobulin Fusion Protein Attenuates Activation of Coagulation, but not of Fibrinolysis, during Lethal Bacteremia in Baboons

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Baboons *(Papio anubis)* receiving a lethal intravenous infusion with live *Escherichia coli* were pretreated with either a 55-kDa tumor necrosis factor (TNF) receptor–IgG fusion protein (TNFR55:IgG) ($n = 4$, 4.6 mg/kg) or placebo ($n = 4$). Neutralization of TNF activity in TNFR55:IgG-treated animals was associated with a complete prevention of mortality and a strong attenuation of coagulation activation as reflected by the plasma concentrations of thrombin–antithrombin III complexes ($P < .05$). Activation of fibrinolysis was not influenced by TNFR55:IgG (plasma tissue-type plasminogen activator and plasmin–α_2-antiplasmin complexes), whereas TNFR55:IgG did inhibit the release of plasminogen activator inhibitor type I ($P < .05$). Furthermore, TNFR55:IgG inhibited neutrophil degranulation (plasma levels of elastase–α_1-antitrypsin complexes, $P < .05$) and modestly reduced release of secretory phospholipase A_2. These data suggest that endogenous TNF contributes to activation of coagulation, but not to stimulation of fibrinolysis, during severe bacteremia.

Tumor necrosis factor-α (TNF) is considered to be a key mediator in the pathogenesis of sepsis syndrome. TNF is released into the circulation early after intravenous bacterial challenges in animals, and neutralization of endogenous TNF prevents lethality in these acute models [1]. The role of TNF in disturbances of the hemostatic mechanism during systemic infection is less clear. Sepsis- or endotoxin-induced activation of the coagulation system is driven by the tissue factor–mediated extrinsic pathway [2]. TNF potently up-regulates tissue factor expression on endothelial and mononuclear cells, suggesting a role for this cytokine in coagulation activation [1, 2]. In accord, intravenous injection of recombinant TNF into humans or baboons induced activation of the common pathway of the coagulation system [3, 4]. However, treatment of baboons with severe bacteremia or endotoxemia with a neutralizing anti-TNF monoclonal antibody did not result in a noticeable effect on activation of the coagulation system, in spite of the fact that such treatment afforded significant protection against lethality [5–7]. Similarly, anti-TNF did not influence coagulation activation during nonlethal endotoxemia in chimpanzees [8]. By contrast, anti-TNF completely prevented the fibrinolytic response to nonlethal endotoxemia [8], whereas in the lethal models fibrinolysis was not investigated.

Recently, the capacity of a 55-kDa TNF receptor–IgG fusion protein (TNFR55:IgG) to effectively neutralize TNF activity and to strongly reduce lethality in baboons infused with a lethal dose of live *Escherichia coli* was reported [9]. Interestingly, treatment with TNFR55:IgG attenuated fibrinogen consumption and the increases in prothrombin and partial thromboplastin times caused by the bacteremia. These findings prompted us to study in more detail the effect of TNFR:IgG on the activation of coagulation and fibrinolysis.

Methods

Study design. The present study was performed simultaneously with a previously reported investigation [9]. Details of the animal study have been reported elsewhere [9]. Briefly, baboons *(Papio anubis)* (10–14 kg) were challenged with *E. coli* with or without pretreatment with TNFR55:IgG at the Research Animal Resource Center of Cornell University Medical College. At time zero, all animals received 10^{10}–10^{11} cfu/kg live *E. coli* (O86:B7) through a femoral venous catheter over 30 min. Baboons were randomized to receive either TNFR55:IgG (Ro 45-2081; 4.6 mg/kg; $n = 4$) or placebo ($n = 4$) as a 15-min intravenous infusion directly prior to infusion of bacteria. Endotoxin concentration of the TNFR55:IgG preparation was <0.4 EU/mg of protein. Arterial blood was obtained at −0.5, 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, and 8 h relative to the infusion of *E. coli.*

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Assays. All assays were performed in heparinized plasma samples and were described in detail previously [4, 10]. Coagulation activation was determined by measuring thrombin–antithrombin III (TAT) complexes by ELISA. Fibrinolytic activation was monitored by measurements of tissue type plasminogen activator (tPA) by ELISA, plasminogen activator inhibitor type I (PAI-1) by ELISA, and plasmin–α_2-antiplasmin (PAP) complexes by RIA. Levels of PAP complexes are expressed as percentage of the level present in normal baboon plasma in which a maximal amount of PAP complexes was generated by a 1-h incubation with an equal volume of urokinase (50 μg/mL) in the presence of 0.4 *M* methylamine. Neutrophil degranulation was determined by measurement of the plasma concentrations of elastase–α_1-antitrypsin complexes by RIA. Secretory phospholipase A_2 ($sPLA_2$) was measured with an ELISA.

Statistical analysis. All values are expressed as mean ± SE. Differences within groups were tested by repeated-measures analysis of variance. Differences between groups were tested by repeated-measures analysis of variance (interaction between treatment and time). *P* values are therefore derived from analyses in which data from all time points were included. $P < .05$ was considered to represent a significant difference.

Results

In the previously reported study, treatment with TNFR55:IgG had been found to completely neutralize TNF activity throughout the observation period and to significantly attenuate the severe hypotensive response and lethality observed in the animals infused with *E. coli* [9]. Three of four baboons pretreated with placebo had died, after 6, 30, and 36 h, respectively, while all baboons treated with TNFR55:IgG survived.

The infusion of *E. coli* was associated with a strong activation of the common pathway of the coagulation system, as reflected by a sustained rise in the plasma concentrations of TAT complexes, peaking after 6 h (959 ± 296 ng/mL; $P < .05$ vs. time) (figure 1). This coagulation response was significantly blunted by treatment with TNFR55:IgG. Peak levels of TAT complexes in TNFR55:IgG-treated animals were 322 ± 90 ng/mL ($P < .05$ vs. placebo). Bacteremia also induced a marked activation of the fibrinolytic system, as indicated by increases in the plasma concentrations of tPA (peak of 12.9 ± 2.2 ng/mL after 8 h; $P < .05$ vs. time), PAI-1 (peak of 3669 ± 1298 ng/mL after 8 h; $P < .05$), and PAP complexes (peak of 0.68% ± 0.32% after 4 h; $P < .05$) (figure 1). Treatment with TNFR55:IgG had no influence on the rise in tPA levels but significantly attenuated the release of PAI-1 during bacteremia. Peak PAI-1 levels in TNFR55:IgG-infused baboons were 1095 ± 314 ng/mL ($P < .05$ vs. placebo). The plasma concentrations of PAP complexes tended to be higher in TNFR55:IgG-treated animals (peak of 0.85% ± 0.32% after 4 h), but the difference compared with control animals did not reach statistical significance.

As reported earlier [9], bacteremia was associated with a sustained neutrocytopenia, which was partially reversed by

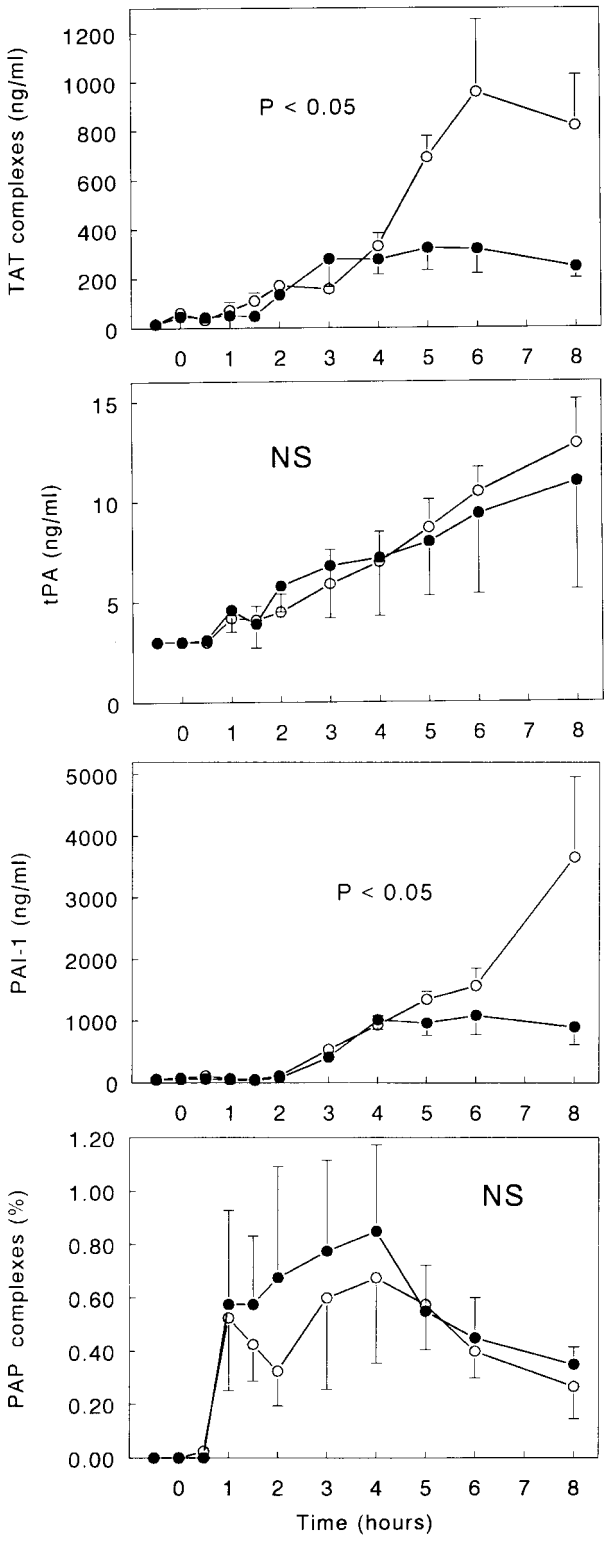

Figure 1. Effect of pretreatment with 55-kDa TNF receptor–IgG fusion protein (TNFR55:IgG) on activation of coagulation and fibrinolysis. Data are mean (±SE) plasma concentrations of thrombin-antithrombin III (TAT) complexes, tissue-type plasminogen activator (tPA), plasminogen activator inhibitor type I (PAI-1), and plasmin–α_2-antiplasmin (PAP) complexes after infusion of *Escherichia coli* in baboons pretreated with either TNFR55:IgG ($n = 4$, ●) or placebo ($n = 4$, ○). *P* indicates difference between groups by analysis of variance. NS = nonsignificant.

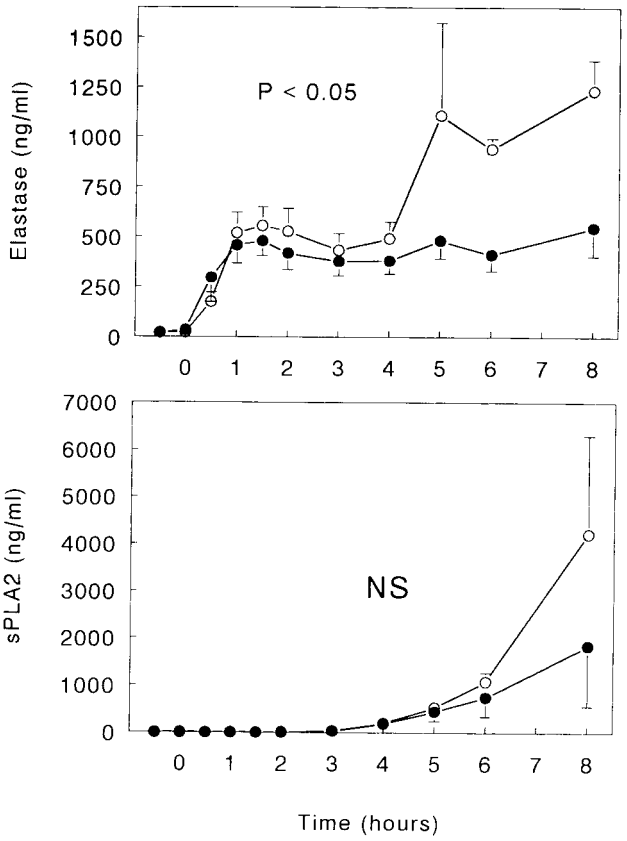

Figure 2. Effect of 55-kDa TNF receptor–IgG fusion protein (TNFR55:IgG) on neutrophil degranulation and secretory phospholipase A2 (sPLA2) release. Data are mean (±SE) plasma concentrations of elastase–α_1-antitrypsin complexes and sPLA2 after infusion of *Escherichia coli* in baboons pretreated with either TNFR55:IgG (n = 4, ●) or placebo (n = 4, ○). P indicates difference between groups by analysis of variance. NS = nonsignificant.

TNFR55:IgG. Infusion of *E. coli* also resulted in degranulation of neutrophilic granulocytes, as reflected by an increase in the plasma levels of elastase–α_1-antitrypsin complexes, maximal levels being measured at the end of the study period at 8 h (1234 ± 153 ng/mL; $P < .05$ vs. time) (figure 2). TNFR55:IgG significantly inhibited this response, with elastase–α_1-antitrypsin complexes reaching an approximately constant level at ~500 ng/mL from 1 to 8 h ($P < .05$ vs. placebo). *E. coli* bacteremia further caused a rise in the plasma concentrations of $sPLA_2$, the highest levels being measured at the end of the observation period (8 h: 4217 ± 2081 ng/mL; $P < .05$ vs. time) (figure 2). Although TNFR55:IgG reduced $sPLA_2$ release (8 h: 1845 ± 1284 ng/mL), the difference with control baboons did not reach statistical significance because of a large interindividual variation.

Discussion

The main finding of this study is that pretreatment of baboons with severe *E. coli* bacteremia with a TNF receptor–IgG fusion protein results in an attenuated procoagulant response, as reflected by inhibition of release of TAT complexes, in the absence of an effect on activation of fibrinolysis. This result is in line with the previously reported inhibition of more rough measures of coagulation activation, such as fibrinogen consumption and prolongation of prothrombin and partial thromboplastin times [9]. However, it should be noted that, thus far, studies in which endogenous TNF was neutralized in primates with either lethal or sublethal bacteremia or endotoxemia did not reveal any effect on activation of the coagulation system [5–8], while anti-TNF did abrogate the fibrinolytic response to low-dose endotoxin in chimpanzees [8]. We do not have an explanation for the apparent discrepancy of the present results with those from earlier studies, other than that the compound used to neutralize TNF activity, a dimeric 55-kDa TNF receptor–IgG fusion protein (which also has affinity for lymphotoxin), was different.

We consider it unlikely that our results are influenced by less-than-complete neutralization of TNF. Plasma TNF activity remained completely neutralized (as determined by the highly sensitive WEHI cytotoxicity assay) in animals treated with TNFR55:IgG throughout the entire observation period [9]. In fact, even lower doses of TNFR55:IgG than used in this study were able to completely neutralize TNF activity [9]. Further, TNFR55:IgG has been found to form stable complexes with TNF, in which aspect it differs from a similar IgG fusion protein containing a dimeric p75 TNF receptor [11]. Further, we consider it unlikely that the large spread in the levels of PAP complexes had an important influence on our main conclusion regarding an unaltered fibrinolytic response in animals treated with TNFR55:IgG. Indeed, in our previous study of low-grade endotoxemia in chimpanzees, anti-TNF completely prevented any rise in tPA and PAP complexes [8, 12], while in the present study, the levels of PAP complexes even tended to be higher in animals in which TNF was effectively neutralized.

Excessive activation of neutrophils may contribute in an important way to tissue injury in sepsis [13]. The plasma concentrations of elastase–α_1-antitrypsin complexes have been used as indicators of neutrophil degranulation in vivo and correlate with mortality rates in patients with sepsis [13]. TNF has been found to trigger neutrophil degranulation in vitro [1] and in humans and baboons in vivo [4, 14]. The present results that TNFR55:IgG reduced the release of elastase in bacteremic baboons are in line with a similar inhibition of elastase release by an anti-TNF antibody in endotoxemic chimpanzees [8] and therefore extend the role of TNF in neutrophil degranulation to lethal bacteremia.

$sPLA_2$ is a regulatory enzyme controlling the synthesis of eicosanoids and platelet-activating factor, which has been implicated in the pathogenesis of tissue injury associated with sepsis. Injection of TNF into baboons elicited a rapid release of $sPLA_2$ [4]. Pretreatment with TNFR55:IgG was associated with a modest, nonsignificant inhibition of $sPLA_2$ release, suggesting that TNF is not a critical mediator of this response in sepsis. Similarly, infusion of an anti-TNF antibody also mod-

estly reduced $sPLA_2$ secretion in an earlier study with bacteremic baboons [15].

One control animal had died at the time the last blood sample was taken (after 8 h). The lack of this sample is unlikely to influence the results, since plasma concentrations of PAI-1, elastase–α_1-antitrypsin complexes, and sPLA2 were higher in more ill animals, and thus the availability of an 8-h sample from the baboon that died early would likely have made differences between control animals and animals treated with TNFR55:IgG more pronounced.

Severe *E. coli* bacteremia in baboons may be a useful model to study pathogenetic mechanisms underlying inflammatory responses during fulminant septic shock. We herein demonstrate that neutralization of endogenous TNF by pretreatment with a 55-kDa TNF receptor–IgG fusion protein not only prevents lethality but also significantly attenuates coagulation activation while not influencing fibrinolysis. Compared with earlier findings in primates with mild endotoxemia, revealing unaltered coagulation activation and inhibited fibrinolytic activation in animals treated with an anti-TNF antibody [8], these data illustrate the divergence of various sepsis models, which depend on the severity and the time course of toxicity evoked by the bacterial insult, and also point to the difference in the effects exerted by the various anti-TNF agents used in such studies.

References

1. Van der Poll T, Lowry SF. Tumor necrosis factor in sepsis: mediator of multiple organ failure or essential part of host defense? Shock **1995**;3:1–12.
2. Levi M, ten Cate H, van der Poll T, van Deventer SJH. New insights in the pathogenesis of disseminated intravascular coagulation in sepsis. JAMA **1993**;270:975–9.
3. Van der Poll T, Büller HR, Ten Cate H, et al. Activation of coagulation after administration of tumor necrosis factor to normal subjects. N Engl J Med **1990**;322:1622–7.
4. Van der Poll T, Jansen PM, van Zee KJ, et al. Tumor necrosis factor α induces activation of coagulation and fibrinolysis in baboons through an exclusive effect on the p55 receptor. Blood **1996**;88:922–7.
5. Hinshaw LB, Tekamp-Olson P, Chang ACK, et al. Survival of primates in LD100 septic shock following therapy with antibody to tumor necrosis factor (TNFα). Circ Shock **1990**;30:279–92.
6. Hinshaw LB, Emerson TE, Taylor FB Jr, et al. Lethal *Staphylococcus aureus*–induced shock in primates: prevention of death with anti-TNF antibody. J Trauma **1992**;33:568–73.
7. Stevens DL, Bryant AE, Hackett SP, et al. Group A streptococcal bacteremia: the role of tumor necrosis factor in shock and organ failure. J Infect Dis **1996**;173:619–26.
8. Van der Poll T, Levi M, Van Deventer SJH, et al. Differential effects of anti-tumor necrosis factor monoclonal antibodies on systemic inflammatory responses in experimental endotoxemia in chimpanzees. Blood **1994**;83:446–51.
9. Van Zee KJ, Moldawer LL, Oldenburg HSA, et al. Protection against lethal *Escherichia coli* bacteremia in baboons *(Papio anubis)* by pretreatment with a 55-kDa TNF receptor (CD120a)–Ig fusion protein, Ro 45-2081. J Immunol **1996**;156:2221–30.
10. Jansen PM, Boermeester MA, Fischer E, et al. Contribution of interleukin-1 to activation of coagulation and fibrinolysis, to neutrophil degranulation and the release of $sPLA_2$ in sepsis. Studies in non-human primates following interleukin-1α administration and during lethal bacteremia. Blood **1995**;86:1027–34.
11. Evans TJ, Moyes D, Carpenter A, et al. Protective effect of 55- but not 75-kD soluble tumor necrosis factor receptor–immunoglobulin G fusion proteins in an animal model of Gram-negative sepsis. J Exp Med **1994**;180:2173–9.
12. Biemond BJ, Levi M, ten Cate H, et al. Plasminogen activator and PAI-1 release during experimental endotoxemia in chimpanzees: effects of various interventions in the cytokine and coagulation cascades. Clin Sci **1995**;88:587–94.
13. Nuijens JH, Abbink JJ, Wachtfogel YT, et al. Plasma elastase–α_1-antitrypsin and lactoferrin in sepsis: evidence for neutrophils as mediators of fatal sepsis. J Lab Clin Med **1992**;19:159–68.
14. Van der Poll T, van Deventer SJH, Hack CE, et al. Effects on leukocytes following injection of tumor necrosis factor into healthy humans. Blood **1992**;79:693–8.
15. Redl H, Schlag G, Schiesser A, Davies J. Tumor necrosis factor is a mediator of phospholipase release during bacteremia in baboons. Am J Physiol **1993**;264:H2119–23.